

DNA methyltransferases: a novel target for prevention and therapy

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Cancer is the second leading cause of death in US. Despite the emergence of new, targeted agents, and the use of various therapeutic combinations, none of the available treatment options are curative in patients with advanced cancer. Epigenetic alterations are increasingly recognized as valuable targets for the development of cancer therapies. DNA methylation at the 5-position of cytosine, catalyzed by DNA methyltransferases (DNMTs), is the predominant epigenetic modification in mammals. DNMT1, the major enzyme responsible for maintenance of the DNA methylation pattern is located at the replication fork and methylates newly biosynthesized DNA. DNMT2 or TRDMT1, the smallest mammalian DNMT is believed to participate in the recognition of DNA damage, DNA recombination, and mutation repair. It is composed solely of the C-terminal domain, and does not possess the regulatory N-terminal region. The levels of DNMTs, especially those of DNMT3B, DNMT3A, and DNMT3L, are often increased in various cancer tissues and cell lines, which may partially account for the hypermethylation of promoter CpG-rich regions of tumor suppressor genes in a variety of malignancies. Moreover, it has been shown to function in self-renewal and maintenance of colon cancer stem cells and need to be studied in several cancers. Inhibition of DNMTs has demonstrated reduction in tumor formation in part through the increased expression of tumor suppressor genes. Hence, DNMTs can potentially be used as anti-cancer targets. Dietary phytochemicals also inhibit DNMTs and cancer stem cells; this represents a promising approach for the prevention and treatment of many cancers.

Keywords: DNMT, colon, pancreas, breast, cancer stem cells, DCLK1

INTRODUCTION

DNA methylation and histone modifications are two key players in epigenetic regulation of gene expression in mammalian cells. Epigenetic modifications play an important role in multi-stage carcinogenesis (1, 2). Extensive analysis of different types of human cancer has revealed that epigenetic alteration of the genome plays a causal role in tumorigenesis. Analysis of epigenetic alterations in tissue samples, together with the histological features of each cancer may aid the understanding of the molecular background of histological heterogeneity in human cancers. DNA methylation, a covalent chemical modification resulting in addition of a methyl group at the carbon 5 position of the cytosine ring in CpG dinucleotides, is one of the most consistent and best known epigenetic events in human cancers. In comparison with normal cells, human cancer cells exhibit global DNA hypomethylation, which can lead to genomic instability, and specific promoter hypermethylation of tumor-suppressor genes, which mediates gene silencing (3). Unlike genetic changes, epigenetic changes can be reversed by pharmacological intervention. Many studies have been focused on understanding the structure and functions of key cellular enzymes that mediate these epigenetic processes and on subsequently developing small molecule inhibitors that target these proteins. In addition, epigenetic alterations are increasingly

recognized as valuable targets for the development of cancer therapies.

Methylation of DNA at 5-position of cytosine, catalyzed by DNA methyltransferases (DNMTs), is the predominant epigenetic modification in mammals. Aberrations in methylation play a causal role in a variety of diseases, including cancer. Recent studies have established that like mutation, methylation-mediated gene silencing often leads to tumorigenesis. Paradoxically, genome-wide DNA hypomethylation may also play a causal role in carcinogenesis by inducing chromosomal instability and spurious gene expression. Since methylation does not alter DNA base sequence, much attention has been focused recently on developing small molecule inhibitors of DNMTs that can potentially be used as anti-cancer agents (4). Novel insights provide startling new information regarding DNMTs, with respect to their roles in cancer and the types of proteins they interact with. This information has forced a new view for the role of DNMTs 1, 2, and 3 (5). In this study, we have reviewed the updated information for DNMTs as novel targets for prevention and therapy.

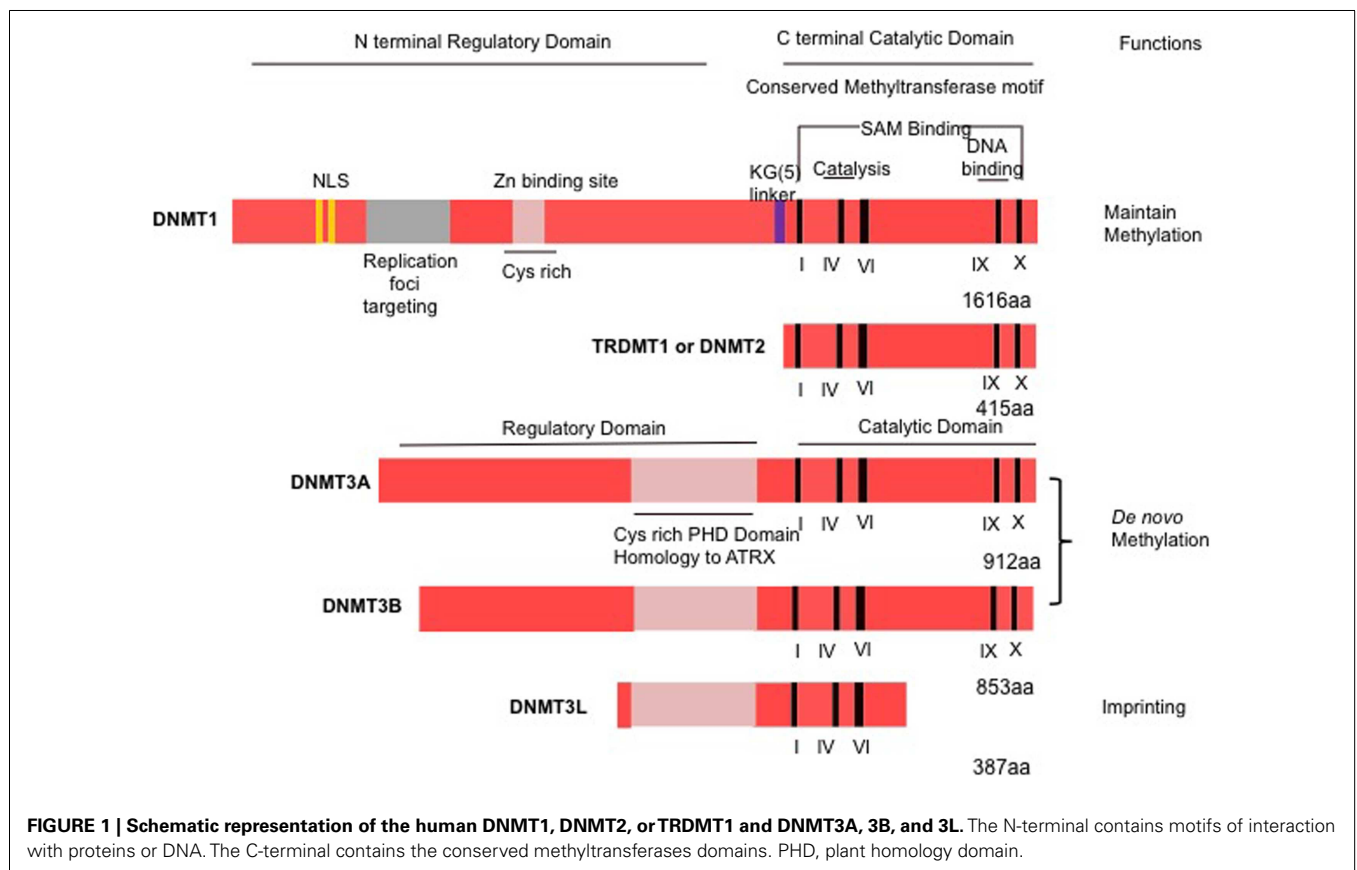
DNA METHYLTRANSFERASES

Methylation of mammalian genomic DNA is catalyzed by DNMTs. Patterns of DNA methylation are established by the coordinated

action of the DNMTs and associated factors, such as the polycomb proteins in the presence of *S*-adenosyl-methionine that serves as a methyl donor (5). The mammalian DNMT family includes four active members: DNMT1, DNMT3A, DNMT3B, and DNMT3L (6, 7). Mammalian DNMTs are responsible for methylation pattern acquisition during gametogenesis, embryogenesis, and somatic tissue development (5). DNMT1 is the most abundant DNMT involved in the maintenance of methylation (8). DNMT3 functions as a *de novo* methyltransferase and consists of two related proteins encoded by distinct genes, DNMT3A and DNMT3B (9). Of special interest is DNMT2, which has the potential to methylate RNA instead of DNA (10) (Figure 1).

The DNMT1 is the major enzyme responsible for maintenance of the DNA methylation pattern. DNMT1 is also often referred to as maintenance methyltransferase, because it is believed to be the primary enzyme responsible for copying methylation patterns after DNA replication (8). DNMT1 is located at the replication fork and methylates newly biosynthesized DNA (4). The mammalian DNMTs comprised two regions: a C-terminal catalytic portion and a large multi-domain N-terminal region of variable size, which encodes regulatory functions. The C-terminal part is composed of 500 amino acids that are conserved between C5 DNMTs of eukaryotes and prokaryotes, and harbor the active center of the enzyme, containing amino acids motifs characteristic of the cytosine-C5 methyltransferases. The N-terminal region generally contains 621 amino acids that are not essential for DNMT1

activity (4), but are required for discriminating between hemi-methylated and unmethylated DNA. The catalytic domains of all the DNMTs share a common core structure, known as “AdoMet-dependent methyltransferase.” This domain is involved in both cofactor binding (motifs I and X) and substrate catalysis (motifs IV, VI, and VIII). A non-conserved region between motifs VIII and IX, believed to be the target recognition domain, is involved in DNA recognition and specificity (Figure 1). DNMT1 is the most abundant DNMT targeted to replication foci. Three sequences in the N-terminal region increase the precision in maintenance of methylation and give the enzyme direct access to the nuclear replication site: the proliferating cell nuclear antigen (PCNA) binding domain, the replication foci targeting sequence, and the poly-bromo homology domain. PCNA is required for DNA replication, and the DNMT1–PCNA interaction may allow the newly synthesized daughter strands to be rapidly remethylated before being packaged into chromatin. This tight association of the DNMT1 with the replication machinery allows DNMT1 to bind newly replicated and the naked DNA (11). Without DNMT, some genes may hinder interaction with the replicating foci. Cell-cycle regulator p21 can disrupt DNMT–PCNA interaction, suggesting that p21 may negatively regulate methylation by blocking access of DNMT to PCNA, particularly during DNA damage when p21 protein is induced. Moreover, p21 can itself inhibit DNMT1 gene expression. Under experimental conditions, DNMT1 has up to a 50-fold preference for hemi-methylated DNA substrate and is localized



to the replication foci during S-phase. It is proposed to duplicate DNA methylation patterns in the daughter strands during DNA replication (12). Mouse models with both alleles of DNMT1 deleted are embryonic lethal at approximately day E9 (13). The retinoblastoma gene product Rb, another cell-cycle regulator protein, can bind to DNMT1 and inhibit its methyltransferase activity during DNA replication in the cell cycle. Loss of functional Rb may grant DNMT1 free access to the genome, which could allow aberrant *de novo* methylation of CpG. These observations point to a complicated network of connections between DNMT1 and several cellular proteins involved in gene regulation and epigenetic signaling during cell replication (4).

DNMT2 is the smallest mammalian DNMT and it is now termed as TRDMT1. It is composed solely of the C-terminal domain, and does not possess the regulatory N-terminal region. The structure of DNMT2 suggests that this enzyme participates in the recognition of DNA damage, DNA recombination, and mutation repair (14). DNMT2 is a methyltransferase homolog that methylates cytosine-38 in the anticodon loop of aspartic acid transfer RNA instead of DNA (15).

DNMT3A and DNMT3B cannot differentiate between unmethylated and hemi-methylated CpG sites, and they cannot copy a specific pattern of methylation or contribute to the maintenance of methylation pattern (16). Because they show no preference for hemi-methylated DNA, both enzymes appear to function as *de novo* methyltransferases (16) and show a disperse distribution throughout the nucleus not associated with replication sites, even during S-phase (17). This finding suggests that these DNMTs utilize a different mechanism for accessing the densely packed chromatin and for interacting with their target sites that may involve auxiliary factors such as chromatin remodeling complexes (18). Mice lacking DNMT3A die at about 4 weeks of age, whereas DNMT3B knockout induces embryonic lethality at E14.5–E18.5 (16). Possessing homology to DNMT3A and DNMT3B, DNMT3L assists the *de novo* methyltransferases by increasing their ability to bind to the methyl group donor, S-adenosyl-L-methionine, and stimulating their activity *in vivo* (19), although DNMT3L has no catalytic activity itself. DNMT3L homozygous-null mice are viable, whereas heterozygous embryos derived from homozygous DNMT3L-null oocytes die around E9 and display impaired maternal methylation imprints and biallelic expression of imprinted genes normally expressed only from the allele of paternal origin (20). Cooperation among different DNMTs is also required in methylating some regions of the genome, particularly repetitive elements. It is widely speculated that DNMT1 acts in “maintenance” of methyltransferase during DNA synthesis and that DNMT3A and DNMT3B act as “*de novo*” enzymes in development. However, mounting evidence indicates that DNMT1 may also be required for *de novo* methylation of genomic DNA and that DNMT3A and DNMT3B contribute to maintain methylation during replication (21).

DNMT3A and DNMT3B are highly expressed in early embryonic cells, the stage in which most programmed *de novo* methylation events occur, are downregulated after differentiation and in adult somatic tissues, and are overexpressed in tumor cells (22). DNMT3B has been shown to play a crucial role in incorporating *de*

*nov*o hypermethylation of promoter CpG islands, a possible mechanism for tumor suppressor gene inactivation within human cancer cells (22). Another member of the DNMT3 family is DNMT3L, a regulatory factor for *de novo* methylation without methylation capacities (23). Its amino acid sequence is very similar to that of DNMT3A and DNMT3B but lacks the residues required for DNMT activity in the C-terminal domain (23). Additional studies are required for the precise role for DNMT3L.

Recent studies suggest that an interaction between DNMT1 and DNMT3B may be vital for the maintenance of patterns of DNA methylation in human colon-cancer cells, particularly in repeat regions and imprinted genes (23). During early embryogenesis, *de novo* DNA methylation is mediated by DNMT3A and DNMT3B associated with DNMT3L. It has recently been reported that in the cell, DNMT3A and DNMT3B are tightly associated with nucleosomes containing methylated DNA (23). Both the direct interaction of these proteins with the histone tails and the polymerization of DNMT3A could contribute to the stable association of these enzymes with chromatin. The levels of DNMTs, especially those of DNMT3A and DNMT3B, are often increased in various cancer tissues and cell lines. This may partially account for the hypermethylation of promoter CpG-rich regions of tumor suppressor genes in a variety of malignancies (24).

DNA METHYLTRANSFERASES OVEREXPRESSION AND CANCER

The human DNMTs 1, 3A, and 3B coordinate mRNA expression in normal tissues and overexpression in tumors (25). The expression levels of these DNMTs are reportedly elevated in cancers of the colon (26), prostate (27), breast (28, 29), liver (30), and in leukemia (31). The role of altered expression of DNMTs in DNA hypomethylation and hypermethylation in cancer is uncertain and may involve changes in mRNA or protein expression. There is considerable evidence indicating an up-regulation of DNMT1 in cancer (32, 33). In addition, experimental evidence indicates that forced overexpression of the murine DNMT1 gene in NIH3T3 cells results in cellular transformation (34). In human fibroblasts, sustained overexpression of DNMT1 leads to the processive time-dependent hypermethylation of a number of CpG islands (35). DNMT1 expression is upregulated following fos overexpression and appears to play a role in the fos-induced cell transformation (36). Conversely, reduction of DNMT1 levels appears to have protective effects. Mice predisposed to colonic polyp formation (Min mice) develop fewer polyps in a DNMT1 heterozygous background. Similar results are seen when treated with 5aza-dC (37). Reduction of DNMT1 through an antisense approach also blocks tumorigenesis (38–41). Interestingly, deletion of the DNMT1 gene in a colon cancer cell line (HCT116), while resulting in slower growth, diminished genomic methylation levels modestly ~20% (42). In particular, aberrant CpG island methylation was retained suggesting that another methyltransferase maintains this methylation. Like DNMT1, the aforementioned DNMT3A and DNMT3B enzymes also appear to be modestly overexpressed in cancer. Therefore, the balance of all three enzymes and their accumulative and coordinated effects must be studied (25, 43). DNMTs and its importance in specific cancers are summarized in **Table 1**.

Table 1 | DNA methyltransferases and its importance in specific cancer.

DNA methyltransferases	Importance in specific cancer	Reference
DNMT1	Leukemia: upregulated – 5.3-fold expression Gastric cancer – 64.8% localized in the cytoplasm and nuclei Breast cancer – 16.6% Hepatocellular carcinoma – 100% Pancreatic cancer – highly expressed – Gli target gene Colon cancer – highly expressed Glioblastoma – overexpressed	Mizuno et al. (44) Yang et al. (45) Girault et al. (28) Nagai et al. (46) He et al. (47) Robertson et al. (25) Rajendran et al. (48)
DNMT2 or TRDM T1	Hepatocellular carcinoma – reduced expression Colorectal and stomach cancers – lower mRNA expression	Saito et al. (49) Kanai et al. (50)
DNMT3A	Acute myeloid leukemia – 22.1% mutations and affect translation Gastric cancer – 70.4% localized in the cytoplasm Breast cancer – 14% Hepatocellular carcinoma – 60% Pancreatic cancer – highly expressed – regulated by Gli1 Colon cancer – highly expressed	Ley et al. (51) Yang et al. (45) Girault et al. (28) Nagai et al. (46) He et al. (47) Robertson et al. (25)
DNMT3B	Leukemia: upregulated – 11.7-fold expression Gastric cancer – 51.9% localized in the cytoplasm Breast cancer – 81.8% poor prognosis Breast cancer cell lines-hypermethylation defect resulted in aberrant – overexpression DNMT activity Hepatocellular carcinoma (60%) and mRNA levels high Colon cancer – highly expressed Prostate cancer – overexpressed Glioblastoma – overexpressed	Mizuno et al. (44) Yang et al. (45) Girault et al. (28) Roll et al. (52) Nagai et al. (46) Robertson et al. (25), Ibrahim et al. (53) Kobayashi et al. (54) Rajendran et al. (48)
DNMT3L	Cervical cancer – promising biomarker Embryonal carcinoma – novel biomarker	Gokul et al. (55) Minami et al. (56)

COLON CANCER

Colorectal cancer is the second leading cause of death in the United States and is a major health problem globally (57). The lifetime risk of developing colorectal cancer in both men and women is about 1 in 20 (5.1%) (58). Colorectal cancer affects over 146,970 individuals yearly, and accounts for around 49,920 deaths (59). The American Cancer Society (ACS) estimated 96,830 new cases (48,450 men and 48,380 women) would be diagnosed with colon cancer during 2014 and also estimated 50,310 deaths (26,270 men and 24,040 women) (60–62). Screening for colon cancer can be done by colonoscopy to find polyps, and removing these polyps at an early stage can prevent cancer progression. When the polyps are allowed to persist in the colon for a long time, they may develop into cancer. Hence, regular colonoscopy is recommended in the United States for those over 50 years of age (63). Recurrence of colon cancer is common, with an estimated 40% of cases returning within 3–5 years of treatment. Chemotherapeutic compounds currently being used for the treatment of colorectal cancer include 5-fluorouracil, Oxaliplatin, and Irinotecan hydrochloride or drug combinations FOLFOX or FOLFIRI. Because conventional therapies, including surgical resection, chemotherapy, and radiation are often inadequate in treating this disease, new treatment options are critically needed. Despite the emergence of novel

targeted agents and the use of various therapeutic combinations, no treatment options are available that are curative in patients with advanced cancer. More recently, the cancer stem-cell concept is gaining importance, because it suggests new approaches to anti-cancer therapies (64, 65). Cancer tissues are composed from several heterogeneous cancer cells and a small population of cancer cells is supposed to have higher tumor-initiating ability. These higher tumorigenic populations are named “cancer stem cells (CSCs)” or “cancer initiating cells (CICs).” CSCs/CICs are defined as small population of cancer cells which has (1) higher tumor-initiating ability, (2) self-renewal, and (3) differentiation (66–71). CSCs/CICs have been reported to be resistant to chemotherapy, radiotherapy, and certain molecular targeting therapies (72); thus, elucidation of the molecular mechanisms of the maintenance of CSCs/CICs should be useful for establishing efficient CSC/CIC targeting treatment. Potential markers of colorectal cancer stem cells have been proposed, including CD133, CD166, CD24, CD44, ALDH1, LGR5, and DCLK1 (65, 73–76). Most recently, Nakanishi and colleagues demonstrated that doublecortin and CaM kinase-like-1 (DCLK1) distinguishes between tumor and normal stem cells in the intestine. Their studies demonstrated that specific ablation of DCLK1⁺ stem cells resulted in a marked regression of polyps without apparent damage to the

normal intestine and could be a therapeutic target for colon cancer (77, 78).

COLON CANCER AND DNMTs

DNMT1 and DNMT3B modulate distinct polycomb-mediated histone modifications in colon cancer (79). Manipulation of DNMT1 levels has been used as a tool to study the effect of DNA hypomethylation on tumorigenesis in several *in vivo* studies. The role of DNA methylation *in vivo* was first explored in the intestine using the *APC^{Min/+}*, a commonly used mouse model of intestinal tumors since it very closely mimics the human familial adenomatous polyposis (FAP) condition (80–82). These mice develop benign intestinal tumors with a rare occurrence of malignant cancers (81). Treatment of *APC^{Min/+}* mice with the demethylating agent, 5-aza-2'-deoxycytidine, significantly reduces tumor formation in the intestine, suggesting that DNA methylation may play an important role during tumorigenesis (80). Moreover, overexpression of DNMT3B1 in the *APC^{Min/+}* model enhanced colorectal carcinogenesis and caused tumor suppressor gene methylation (83). Other studies have also shown that crossing *APC^{Min/+}* mice with DNMT1 hypomorphic mice results in complete suppression of macroscopic intestinal neoplasia (84). Reduced DNMT1 expression also affects the frequency of malignant intestinal tumors in DNA mismatch repair deficient mice (*Mlh1^{-/-}*) (85, 86).

COLON CANCER STEM CELLS AND DNMTs

Previous studies have reported that hematopoietic stem cell self-renewal can be abrogated by conditional gene knockout of DNMT1, while the mature differentiated hematopoietic lineage is not affected (87). DNMT1 is essential for maintenance of the leukemia stem cells of bilinear myeloid-B lymphoid leukemia induced by transduction of c-Myc and Bcl-2 (88). DNMT1 was also shown to be essential for the self-renewal of skin progenitor cells (89). Most recently, studies have demonstrated that DNMT1 functions in the maintenance of human colon CSCs/CICs using the human colon cancer cell line HCT116 and its DNMT1 somatic knockout variant (*DNMT1^{-/-}*). The rates of CSCs/CICs were evaluated by side population (SP) analysis, ALDEFUOR assay, and expression of CD44 and CD24. SP, ALDEFUOR⁺ and CD44⁺ and CD24⁺ (CD44⁺CD24⁺) cell rates were lower in *DNMT1^{-/-}* cells than in control HCT116 cells. Since CSCs/CICs have higher tumor-initiating ability than that of non-CSCs/CICs, the tumor-initiating ability was also addressed by injecting immune deficient NOD/SCID mice (90). *DNMT1^{-/-}* cells showed less tumor-initiating ability than did control HCT116 cells, whereas the growing rate of *DNMT1^{-/-}* cells showed no significant difference from that of HCT116 cells both *in vitro* and *in vivo* (90). Similar results were obtained for cells in which DNMT1 had been transiently knocked-down using gene-specific siRNAs. These results indicate that DNMT1 is essential for maintenance of colon CSCs/CICs and that short-term suppression of DNMT1 might be sufficient to disrupt CSCs/CICs (90).

PANCREATIC CANCER

Pancreatic cancer is an aggressive malignancy with one of the highest mortalities among all cancers. It is the fourth leading cause of cancer death in the United States with <5% 5-year survival rate.

The lifetime risk of developing pancreatic cancer in both men and women is about 1 in 79 (1.27%) (91). The ACS estimated that new cases of 46,420 Americans (23,530 men and 22,890 women) would be diagnosed with pancreatic cancer during 2014. The ACS also estimated that 39,590 Americans (20,170 men and 19,420 women) would die of pancreatic cancer in 2014 (60, 62). Despite advances in molecular pathogenesis, problems such as drug resistance and susceptibility for metastasis make pancreatic cancer a major unsolved health problem in the United States (92). Unfortunately, pancreatic cancer is a rapidly invasive, metastatic tumor that is resistant to standard therapies (93). At present, single agent-based chemotherapy (e.g., gemcitabine) is the mainstay treatment for metastatic pancreatic adenocarcinoma. Recent data indicate that in addition to Gemcitabine and a 5-FU plus, a platinum agent such as Oxaliplatin could be used as a therapeutic paradigm for early-stage cancer patients (94). However, none of the available current chemotherapeutic agents have objective response rates of over 10% (95, 96). The magnitude of this problem mandates the need for novel therapeutic agents. Recently, CSCs and epithelial–mesenchymal transition (EMT)-type cells, which share molecular characteristics with CSCs, have been postulated to play critical roles in drug resistance and cancer metastasis in pancreatic cancer (97). Recent studies suggest that CD44⁺CD24⁺ESA⁺ (epithelial specific antigen) and ALDH1 could potentially be pancreatic cancer stem-cell markers (98, 99). In addition, we have determined that an identified intestinal stem-cell marker DCLK1 is also expressed in a small proportion of cells in the pancreas and in pancreatic cancer stem-cell marker (100, 101).

PANCREATIC CANCER AND DNMTs

Aberrant DNA hypermethylation patterns have been observed in both early- and late-stage human pancreatic tumors (86, 102). Oghamian et al. hypothesized that reduction in DNA methylation levels may decrease pancreatic tumor burden *in vivo* (86). Mice heterozygous for mutation of APC gene are predisposed to the development of benign intestinal polyps, whereas mice homozygous for a mutation in the *Trp53* gene develop a wide range of malignancies, including sarcomas and lymphomas (103). The combined mutation of *APC^{Min/+}* and *Trp53^{-/-}* has been shown to result in a shift in phenotype with nearly 83% of the animals developing abnormalities of the exocrine pancreas, of which 22% also possessed pancreatic acinar cell carcinoma (104, 105). Oghamian et al. also studied the role of DNA methylation in pancreatic tumorigenesis, using this *APC^{Min/+} Trp53^{-/-}* mouse model of exocrine pancreatic cancer and crossed it with mice carrying hypomorphic alleles of DNMT1. They found that tumor burden, but not tumor size, is significantly reduced with decreasing DNMT1 levels, suggesting that DNA methylation is involved in pancreatic tumorigenesis in this mouse model. Their detailed analyses also showed that the reduction in tumor burden is the result of a decrease in both early- and late-stage lesions. In addition, they observed decreased levels of DNA methylation at candidate genes in the normal pancreas of DNMT1 hypomorphic mice (86). Moreover, the expression of DNMT1 protein increased with the development of pancreatic cancer from normal tissue to precancerous lesions (PanINs) and to cancer (PDAC) (106–109). Clinic pathological analyses by Wang and colleagues suggested

that PDAC patients with higher DNMT1 protein expression had an overall lower survival rate than those with lower expression. Moreover, higher DNMT1 expression correlated with advanced stages of the disease, reflecting the malignancy potential of PDAC (109). Recent studies examining DNMT mRNA expression in pancreatic cancer has also demonstrated that the levels of the three DNMTs increased with the development of pancreatic cancer from normal duct to pancreatic intraductal neoplasia and further to PDAC. In a statistical study with TNM staging and history of chronic pancreatitis, DNMT3A and DNMT3B, but not DNMT1 expression, correlated with tumor size. Patients with higher levels of DNMT3A, and/or DNMT3B expression had an overall lower survival than those with lower levels of expression (110). Furthermore, univariate analysis showed that high expression levels of DNMTs, in concert with alcohol consumption, tumor differentiation, and TNM staging were statistically significant risk factors. Multivariate analyses showed that high level of DNMT3B expression and tumor differentiation were statistically significant independent poor prognostic factors. Their results suggested that pancreatic carcinogenesis involves an increased mRNA expression of three DNMTs, and they may become valuable diagnostic and prognostic markers as well as potential therapeutic targets for pancreatic cancer (110). Moreover, immunohistochemistry result suggested the expressions of GLI1, DNMT1, and DNMT3a in pancreatic cancer tissues were higher than those in adjacent normal tissues. DNMT1 and DNMT3a are regulated by GLI1 in pancreatic cancer (47).

BREAST CANCER

Breast cancer is the most common form of cancer diagnosed in women worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% of the cancer deaths in 2008 (111, 112). Although the rate of mortality as a result of breast cancer has decreased in western countries, including the USA, in part due to early detection. ACS estimates that 235,030 new breast cancer cases will be identified in the United States in 2014 with an estimated death rate of 40,430 (15%) (60, 62). Breast cancer has a heterogeneous predisposition at both the histological and molecular levels. At least six distinct subtypes have been described on the basis of gene expression profiling, with the most important determinants of these subtypes being the presence or absence of expression of the estrogen or progesterone receptor or the amplification/overexpression of the HER2/ERBB2 locus (113). Subgroups of breast cancer are frequently distinguished into luminal A (estrogen/progesterone-positive), luminal B, HER2⁺, and so-called “triple negative” subtypes (114). Despite the ability of these subtypes to predict outcome, patient response to chemotherapy or targeted therapy remains variable. The current standard of therapy for breast cancer includes surgical resection, radiation, and chemotherapeutic agents such as cisplatin, paclitaxel, carboplatin, bevacizumab, doxorubicin, cyclophosphamide, docetaxel, and epirubicin (115).

Our understanding of CSCs comes primarily from studies on breast cancer stem cells (BrCSC). These have been isolated from human breast tumors or breast cancer-derived pleural effusions using flow cytometry for a specific pattern of cell surface marker expression (CD44⁺, CD24⁻/low, and ESA⁺) (116–118).

Many groups have attempted to confirm that the minimum surface phenotype for a tumorigenic BrCSC is CD44⁺CD24⁻ (119). In addition, CD133 (also a marker of CSC population in other tumors) and in some cases, selected members of the integrin family of receptors (beta1, alpha6, or beta3 integrins), alone or in conjunction with the CD44⁺/CD24⁻ phenotype have also been used to isolate the BrCSCs (120). Aldehyde dehydrogenase (ALDH) expression has similarly been used as a marker for BrCSCs (121). While these markers have exciting implications, it remains to be seen whether a single cell isolated by this method can develop new tumors in animal models.

BREAST CANCER AND DNMTs

In cancer, DNMTs are overexpressed in various tumor types, including breast cancer (28, 52, 122). Surprisingly, the mean levels of DNMT1, DNMT3a, and DNMT3b overexpression have turned out to be quite similar among different tumor types. The DNMT3b gene has shown the highest range of expression (81.8 for DNMT3a compared with 16.6 and 14 for DNMT1 and DNMT3a, respectively). In breast cancer, ~30% of patients revealed overexpression of DNMT3b in the tumor tissue as compared to normal breast tissue. Taking only these overexpressing tumors into account, the DNMT3b expression change was 82-fold, thus being significantly higher (28). Interestingly, DNMT1 and DNMT3a were overexpressed in only 5 and 3% of breast carcinomas (52). Thus, DNMT3b plays the predominant role over DNMT3a and DNMT1 in breast tumorigenesis. This is consistent with a recent study in breast cancer cell lines, which demonstrated a strong correlation between total DNMT activity and overexpression of DNMT3b, but not with the expression of DNMT3a or DNMT1 (52).

ROLE OF DNMT's IN OTHER CANCERS

One study with prostate cancers demonstrated that the activity of DNMT1, DNMT3a, and DNMT3b are twofold to threefold higher in cancer cell lines and cancer tissues, as compared with a benign prostate epithelium cell line and benign prostatic hyperplasia tissues (27). Similarly, in a study with kidney cancer, expression of DNMT1 was higher in the carcinoma tissue compared to the adjacent normal tissue (123). DNMT1 and DNMT3b were also observed to be up to threefold higher in ovarian cancer cells than in normal ovarian surface epithelial cells (124, 125). Similarly, in hepatocellular carcinomas, there is increased expression of DNMT1, DNMT3a, and DNMT3b and a progressive increase in the number of methylated genes from normal liver, chronic hepatitis/cirrhosis to hepatocellular carcinoma. Furthermore, increase in the DNMT3a and DNMT3b mRNA in the carcinoma tissues relative to their non-cancerous normal may be a predictor of poor survival (30). Moreover, Mutze et al. had shown that DNMT1 is a predictive biomarker and potential therapeutic target for chemotherapy in gastric cancer (126). Another study demonstrated that DNMT1 and DNMT3b are overexpressed in gliomas (48).

Similarly in acute myelogenous leukemias, DNMT1, DNMT3a, and DNMT3b levels were significantly upregulated when compared to control bone marrow cells. Although CML cells in the chronic phase did not show significant changes, cells in the acute phase also showed increased levels of the three DNMTs.

These data suggest that upregulated DNMTs may contribute to the pathogenesis of leukemia by inducing aberrant regional hypermethylation (44).

LACUNAS IN THE DNMT's RESEARCH IN CANCER BIOLOGY

DNMT2 or TRDMT1 and DNMT3L are yet to be studied in detail in the different cancers. DNMT2 was shown to methylate tRNA (15, 127, 128). DNMT2 levels were found to be significantly lower in colorectal and stomach cancers than in non-cancerous tissue (50). In addition, significant overexpression of DNMT3b and reduced expression of DNMT2 were observed in hepatocellular carcinomas compared with the corresponding non-cancerous liver tissues (49). DNMT2 utilizes a DNMT mechanism for RNA methylation (129). DNMT2 activity can be monitored by tRNA^{Asp} methylation analysis and has been identified as a promising candidate biomarker for azacytidine therapy (129, 130).

DNMT3L is essential for the establishment of maternal genomic imprints but lacks key methyltransferase motifs and is possibly a regulator of methylation rather than an enzyme that methylates DNA (128, 131). Gokul et al. revealed that significant DNA methylation differences within the promoter of DNMT3L. A regulator of *de novo* DNMTs, DNMT3a and DNMT3b, DNMT3L promoter was found to have lost DNA methylation to varying levels in 14 out of 15 cancer cervix samples that were analyzed. This study highlights the importance of DNA methylation profile at DNMT3L promoter as a promising biomarker for cervical cancer and provides insight into the possible role of DNMT3L in cancer development (55). Another study demonstrated that DNMT3L is a novel marker and was essential for the growth of human embryonal carcinoma (56). Additional studies are required for the precise role for DNMT3L on other cancers.

DNMTs PROMOTE CANCER

Three DNMTs encoded by different genes are known to participate in generating and maintaining the DNA methylation pattern: DNMT1 (132), DNMT3a, and DNMT3b (133). Several studies have shown that DNMT1 is particularly involved in controlling cell growth (134, 135). Chik and Szyf determined whether specific inhibition of DNMT1 would increase the selectivity toward inhibition of cellular transformation and reduce the risk of increasing cell invasiveness. They found that depletion of DNMT1 had the strongest effect on colony growth suppression in cellular transformation but did not induce cellular invasion in MCF-7 and ZR-75-1 non-invasive breast cancer cell lines (136). Studies have also shown that overexpression of DNMT1 in non-transformed cells leads to cellular transformation (34), whereas knockout of DNMT1 protects mice from colorectal cancer (80). In addition, targeted deregulation of DNMTs by hepatitis B virus X protein promotes both specific regional hypermethylation and global hypomethylation. These epigenetic modulations by hepatitis B virus X protein may suggest a mechanism for epigenetic tumorigenesis during HBV-mediated hepatocarcinogenesis (137). Moreover, recent study demonstrated that interleukin (IL)-23, was shown to induce DNMT1 in a STAT5-dependent manner (138).

INHIBITION OF DNMTs FOR ANTI-CANCER THERAPY

Inhibition of DNMTs correlates with reduction in tumorigenicity and increased expression of tumor suppressor genes (139). Hence,

DNMTs are considered valuable targets for the design of specific anti-cancer strategies. The three most commonly used catalytic inhibitors of DNMTs are the nucleoside analogs 5-azaC, 5-azaCdR, and zebularine. The mechanism of action of these three inhibitors is unique. These agents are pro-drugs that need to be incorporated into DNA to act as inhibitors of DNMTs. The nucleoside analogs are first phosphorylated to the triphosphate nucleotide and incorporated into DNA during DNA synthesis. DNMT1 forms a covalent bond with the carbon at position 6 of the cytosine as well as 5-aza-cytosine ring. Under normal conditions the enzyme transfers the methyl group from SAM to the fifth carbon position of the cytosine ring. This enables the release of the enzyme from its covalent bond with cytosine. When a 5'-aza-cytosine ring replaces cytosine in the DNA, the methyl transfer does not take place and the DNMT is trapped on the DNA (140). The replication fork progresses in the absence of DNMT1 resulting in passive loss of DNA methylation in the nascent strand but not the template. Zebularine is a nucleoside analog, which unlike 5-azaC is chemically stable and is orally bioavailable. Zebularine has been originally identified as a cytidine deaminase inhibitor (128, 141). Decitabine and 5-azacytidine are two well-known inhibitors of DNMTs that are effective against bone marrow disorders such as myelodysplastic syndrome (142). There are several DNMTs inhibitors that were studied in several cancers and these are summarized in **Table 2**.

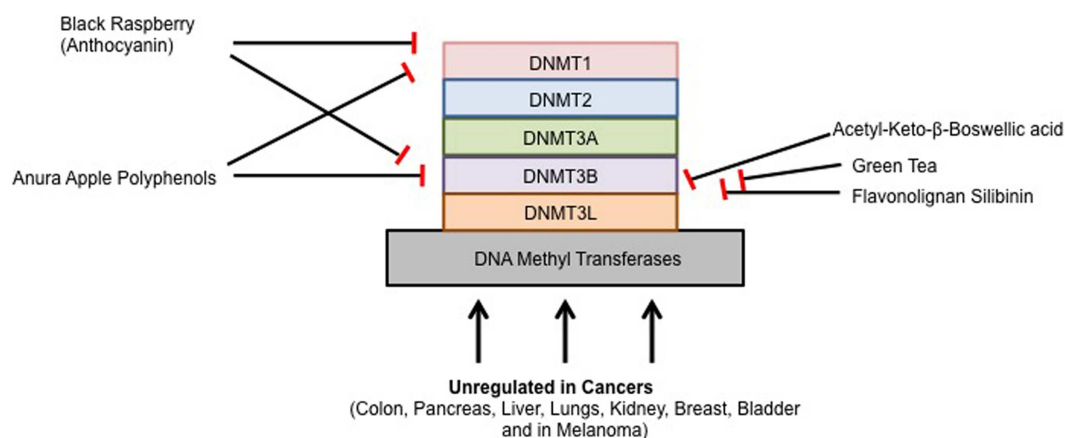
CHEMOPREVENTIVE AGENTS AND DNMTs

To prevent, the onset of cancer, the National Institutes of Health (NIH) in the United States recommended a high fiber, low fat diet, consisting of more fruits and vegetables. Epidemiological studies suggest that diet plays a major role in the prevention of many cancers. The inhibitory effect of chemopreventive compounds on various DNMTs is shown in **Figure 2**.

Black Raspberry-derived anthocyanins were first shown to inhibit DNMT1 and DNMT3B in colon cancer cells. Oral administration of black raspberry powder (BRP) decreased promoter methylation of tumor suppressor genes in tumors from patients with colorectal cancer. The anthocyanins in black raspberries are responsible, at least in part, for their cancer-inhibitory effects. Three days of treatment with anthocyanins suppressed both expression and activity of DNMT1 and DNMT3B proteins in HCT116, Caco2, and SW480 cells. Promoters of CDKN2A, and SFRP2, SFRP5, and of WIF1, an inhibitor of the Wnt pathway, were demethylated by anthocyanins. Moreover, expression of β -catenin and c-Myc mRNA, downstream of Wnt pathway were decreased resulting in reduced cell proliferation and increased apoptosis. Anthocyanins were taken up into HCT116 cells and were differentially localized with DNMT1 and DNMT3B in the same cells visualized using confocal laser scanning microscopy. Although a previous study reported that DNMT3B is regulated by c-Myc in mouse lymphoma, DNMT3B did not bind with c-Myc in HCT116 cells. These findings suggest that anthocyanins are responsible, at least in part, for the demethylation effects of whole black raspberries in colorectal cancers (155). In another study, the biopsies of adjacent normal tissues and colorectal adenocarcinomas were taken from 20 patients before and after oral consumption of BRP (60 g/day) for 1–9 weeks. Methylation status of promoter regions of five tumor suppressor genes was quantified. Protein expression

Table 2 | Available DNMT inhibitors and cancer.

DNMT inhibitors	Dose range	Clinical trials	Reference
5-Azacytine	μM	Phase I, II, III: hematological malignancies	Kaminskas et al. (143), Yoo and Jones (144)
5-Aza-2'-deoxycytidine	μM	Phase I, II, III: hematological malignancies; cervical, non-small-cell lung cancer	Yoo and Jones (144), Prakash et al. (145), Pauer et al. (146)
5-Fluoro-2'-deoxycytidine	μM	Phase I	Yoo and Jones (144), Eidinoff and Rich (147)
5,6-Dihydro-5-azacytidine	μM	Phase I, II: ovarian cancer and lymphomas	Yoo and Jones (144), Curt et al. (148)
Zebularine	μM –mM		Cheng et al. (141), Yoo and Jones (144), Holleran et al. (149)
Hydralazine		Phase I: cervical cancer	Yoo and Jones (144), Zambrano et al. (150)
Procainamide		Preclinical	Yoo and Jones (144), Chuang et al. (151)
EGCG		Preclinical	Yoo and Jones (144), Fang et al. (152)
RG108		Preclinical	Yoo and Jones (144), Brueckner et al. (153)
SGI-110		Phase II: AML	Gros et al. (154)

**FIGURE 2 | Chemopreventive agents and DNMTs.**

of DNMT1 and genes associated with cell proliferation, apoptosis, angiogenesis, and Wnt signaling were measured. They found that the methylation of three Wnt pathway inhibitors, SFRP2, SFRP5, and WIF1, and PAX6a, a developmental regulator, was modulated in a protective direction by BRBs in normal tissues and in colorectal tumors. Moreover, this effect was only observed in patients who received BRB treatment for an average of 4 weeks, but not in the 20 patients who received treatment for 1–9 weeks. This was associated with decreased expression of DNMT1. BRBs modulated expression of genes associated with Wnt pathway, proliferation, apoptosis, and angiogenesis in a protective direction. Their data provide evidence of the ability of BRBs to demethylate tumor suppressor genes and to modulate other biomarkers of tumor development in the human colon and rectum (156).

Anurca apple is an apple variety from southern Italy that is rich in polyphenols. Anurca apple polyphenol extracts inhibited the expression of DNMT1 and DNMT3b in colon cancer cells (157). Furthermore, a recent study demonstrated that

acetyl-keto- β -boswellic acid, derived from the plant *Boswellia serrata*, an Indian frankincense inhibits DNMT activity in colorectal cancer cell lines (158). Most recently, the flavonolignan silibinin, which is the main pharmacologically active component of the milk thistle plant (*Silybum marianum*) with anti-cancer properties is able to significantly inhibit DNMT activity in colon cancer cells (159).

Green tea has also been shown to modulate DNMT activity to inhibit tumorigenesis. EGCG, the major polyphenol in green tea, has many interesting activities and is believed to be a key active ingredient. It dose-dependently inhibited DNMT activity, showing competitive inhibition with a K_i of $6.89 \mu\text{M}$ in human esophageal squamous cell carcinoma cell lines (152, 160). Moreover, a breast cancer study demonstrated that tea polyphenols [catechin, epicatechin, and (–)-epigallocatechin-3-O-gallate (EGCG)] and bioflavonoids (quercetin, fisetin, and myricetin) inhibited DNMT1-mediated DNA methylation in a dose dependent manner. The IC_{50} values for catechin, epicatechin, and

various flavonoids ranged from 1.0 to 8.4 μM , but EGCG was a more potent inhibitor, with IC₅₀ values ranging from 0.21 to 0.47 μM in MCF-7 and MDA-MB-231 cell lines (161, 162). Similarly, genistein present in soybean modulates enzymes that regulate DNA methylation and reactivates tumor suppressor genes in esophageal cancer cells (163, 164). Furthermore, two common catechol-containing coffee polyphenols such as caffeic acid and chlorogenic acid inhibits DNMT1 levels in breast cancer cells. The IC₅₀ values of caffeic acid and chlorogenic acid were 3.0 and 0.75 μM , respectively (161). Curcumin, the active ingredient in turmeric has also been shown to affect DNMT activity. Virtual screening using a DNMT1 homology model suggested two binding modes for curcumin in the catalytic domain. Experimental inhibition of the bacterial C5 DNMT M. SssI confirmed that curcumin and derivatives can inhibit the protein with IC₅₀ values around 30 nM. Curcumin at doses higher than 3 mM also induced a decrease of global DNA demethylation of leukemia MV4-11 cells (154, 165). Furthermore, a recent study demonstrated that curcumin down-regulates DNMT1 expression in AML cell lines, both *in vitro* and *in vivo*, and in primary AML cells *ex vivo* (166).

Thymus serpyllum (wild thyme) is an aromatic medicinal plant possessing several biological properties including anti-cancer activity. *T. serpyllum* extract induced significant cytotoxicity in breast cancer cells (MCF-7 and MDA-MB-231) but not in normal cells. It also induced apoptosis and inhibited the DNMT and HDAC activities in MDA-MB-231 cells (167). Recently, an identification of Kazinol Q, a natural product from formosan plants, was found to act as an inhibitor of DNMTs (168). In addition, a more recent study demonstrated that natural compounds such as EGCG, genistein, withaferin A, curcumin, resveratrol, and guggulsterone inhibit DNMT1, DNMT3a, and DNMT3b expression in breast cancer cell lines (169).

CONCLUSION

The cancer stem cell hypothesis is gaining acceptance after the accumulation of extensive research evidence suggesting that the small subset of the tumor mass is responsible for the sustained growth of the tumor. Furthermore, it is becoming apparent that the cancer stem cells are responsible for disease relapse and resistance to the existing therapies. DNMTs have also upregulated in various cancers including colon, pancreatic, and breast cancers and their stem cells. Current DNMT inhibitors such as Decitabine and 5-azacytidine have adverse side effects. Identifying new drugs that can specifically target DNMTs and also cancer stem cells could lead to a new generation of anti-cancer medicines and with it, a new strategy for treatment. For instance, dietary phytochemicals are natural products found in our diet and can be used to target cancer stem cells. Thus, identification of such cancer stem cell targeting therapy and their use in combination with standard DNMT inhibitors will curtail this dreadful disease.

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